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Genetic Toxicology and Environmental Mutagenesis

The VITOTOX® test, an SOS bioluminescence *Salmonella typhimurium* test to measure genotoxicity kinetics

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Abstract

A new test to detect genotoxicity, that we refer to as the VITOTOX® test, was developed. Four gene fusions that are based on the *Escherichia coli rec*N promoter were constructed and evaluated for their SOS response-dependent induction. The wild-type *rec*N promoter, a derivative mutated in the second LexA binding site, a derivative with a mutated −35 region, and a derivative from which both the second LexA binding site and the −35 region were mutated, were cloned upstream of the promoterless *Vibrio fischeri lux*CDABE operon of pMOL877, in such a way that *lux* became under transcriptional control of the *rec*N promoter derivatives. The inducibility by the SOS response of the promoter constructs was tested in both *E. coli* and in the Ames test *Salmonella typhimurium* strains TA98, TA100 and TA104. In all strains, the highest sensitivity and induction was observed with the plasmids pMOL1067 and pMOL1068, that contain the *lux* operon under control of the *rec*N promoter mutated in the second LexA binding site, or a *rec*N promoter with a mutated −35 region, respectively. Therefore, strains containing pMOL1067 or pMOL1068 were further used for genotoxicity testing. With the VITOTOX test, genotoxicity was detected within 1–4 h. The VITOTOX test is very sensitive: for most products tested, the minimal detectable concentration (MDC) values were considerably lower (5 to > 100 times) than those described for the Ames test and the SOS chromotest. A good correlation was observed with the results from the Ames tests, but certain PAHs that are not mutagenic in the Ames test were genotoxic in the VITOTOX test. With the VITOTOX strains, the kinetics of SOS induction can be determined. This feature made it possible to distinguish between compounds in mixtures of genotoxic products so long as they had different induction kinetics.

Keywords: Ames test; SOS chromotest; VITOTOX® test ; Mutagenicity testing; SOS response; *rec*N

1. Introduction

A strong correlation has been shown to exist between the ability of chemicals to be genotoxic and mutagenic in bacteria and their mutagenic and tumor-initiating properties in mammals [1,2]. Because bacterial short-term tests to detect genotoxic agents are among the simplest, quickest and cheapest to conduct, they are playing an important role in the screening and legislation of genotoxic agents [3,4]. This role is still increasing. Since 1973, many bacterial short-term tests have been proposed, from which the most important are the Ames test, also called the

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Salmonella/microsome assay [5–8], the SOS chromotest [9], the Umu test [10] and the Mutatox test [11].

Undoubtedly, the most popular short-term assays for the identification of mutagens are the Ames test and the SOS chromotest. Experimental results for both tests using 452 compounds have been compared and showed a concordance of 82% [12]. Other studies including these two tests and the Umu test showed that the Ames test could be ranked as the most sensitive method more often than the others. The results also indicated that the Umu test was statistically equivalent to the Ames test [13]. Since the Umu test was the least expensive of the assays, it was recommended as the most suitable for screening large numbers of environmental samples. However, the Ames test strains are still recommended for the performance of bacterial mutation assays: at the International Workshop on the Standardization of Genotoxicity Test Procedures, there was a consensus agreement that the bacterial test battery should consist of *Salmonella typhimurium* TA1537, TA1535, TA98 and TA100, and that the 3 strains TA97a, TA97 and TA1537 could be used interchangeably [14].

A disadvantage of the above-mentioned tests is that none of them can be used to measure the kinetics of induction of the SOS system as a function of genotoxicity, although this can be an important parameter for the comparison of different genotoxic compounds. We therefore decided to construct a battery of test strains that combine features of the Ames test, the SOS chromotest and the Umu test: the constructs must function in the *S. typhimurium* Ames test strains, allowing the simultaneous determination of mutagenic effects by the normal Ames test procedure; they must have an easily detectable phenotype in the presence of genotoxic products to determine the kinetics of genotoxicity; they should also allow toxic products to be detected in a simple way; and they should provide an answer in a short period of time, e.g., within 4 h.

For this purpose four SOS-responsive gene fusions were constructed, using the promoter-less *lux* operon derived from *V. fischeri*, which has an easily detectable phenotype, as reporter system. The constructs were based on the *E. coli rec*N promoter or mutants of this promoter. The *rec*N promoter was chosen primarily because *rec*N is known for its tight regulation by the LexA repressor, but also because the RecN protein becomes the major constituent of the cell after induction of the SOS response [15,16]. All constructs were tested in *E. coli* and in several Ames test *S. typhimurium* strains.

2. Materials and methods

2.1. Strains and growth conditions

The bacterial strains and plasmids used for this work are listed in Table 1. All strains were grown on 869 medium. For genotoxicity testing with the luminescent strains 869 medium was used, containing per liter: tryptone (10 g), yeast extract (5 g), NaCl (5 g), glucose (1 g) $CaCl_2 \cdot 2H_2O$ (0.345 g) and cysteine (30 mg). For mutagenicity testing using the classical Ames test, the minimal medium described by Maron and Ames [7] was used. If required, tetracycline (20 μg/ml) was used to select for the presence of pMOL877 and its derivatives, while ampicillin (100 μg/ml) was used to select for pKM101.

2.2. Molecular cloning techniques and construction of the *rec*N-*lux*CDABE *fusions*

The *rec*N promoter region, that is part of the *E. coli rec*N gene [17], contains two LexA binding sites. One LexA binding site overlaps with the −35 region while the second overlaps with the −10 region and the transcription start point of the *rec*N promoter. To amplify the *rec*N promoter fragment, the following primers were used:

Primer recN1 (*Eco*RI restriction site) recognizes the *rec*N sequence from positions 21 to 49: ACCCC-CTCTCTG*GAATTC*GATTACCCTGG.

Primer recN2 (*Eco*RI restriction site) recognizes the complementary *rec*N sequence from positions 553 to 526: CCATGTC*GAATTC*AGCGCGACCAC-CGAG.

To eliminate the LexA2 binding site from the promoter sequence, primer recN3 was chosen complementary to positions 360 to 338, with sequence AAA*GAATTC*TTATTGTGTACAGTATAAACTG-G, that modifies the sequence of the last three base pairs of the LexA2 binding site (underlined), CAG,

Table 1
Bacterial strains and plasmids

Bacterial strain / plasmids	Relevant characteristics	Reference or origin
Bacterial strains		
E. coli		
ED8739	*sup*E, *hsd*S, *met*, *sup*F, as *E. coli* 8739	[18]
S. typhimurium		
TA98	*his*D3025, *rfa*, *bio*, Δ*uvr*B, pKM101	[7]
MA980	as TA98, containing pMOL1067	This work
MA985	as TA98, containing pMOL1068	This work
TA100	*his*G46, *rfa*, *bio*, Δ*uvr*B, pKM101	[7]
MA982	as TA100, containing pMOL1067	This work
MA988	as TA100, containing pMOL1068	This work
TA104	*his*G428, *rfa*, *bio*, Δ*uvr*B, pKM101	[21]
MA999	as TA104, containing pMOL1066	This work
MA984	as TA104, containing pMOL1067	This work
MA989	as TA104, containing pMOL1068	This work
MA1001	as TA104, containing pMOL1069	This work
Plasmids		
pKM101	Amp^R, involved in enhancement of UV and chemical mutagenesis	[22]
pMOL877	Tc^R, RSF1010 based expression vector containing the promoterless *lux*CDABE operon of *V. fischeri* as reporter system	This work
pMOL1066	pMOL877 in which the wild-type *rec*N promoter of *E. coli* (*rec*N1-2) is cloned upstream of *lux*CDABE (	This work
pMOL1067	pMOL877 in which the LexA2 mutated *rec*N promoter of *E. coli* (*rec*N1-3) is cloned upstream of *lux*CDABE	This work
pMOL1068	pMOL877 in which the promoter up mutation of the *E. coli rec*N (*rec*N2-4) promoter is cloned upstream of *lux*CDABE	This work
pMOL1069	pMOL877 in which the promoter up plus LexA2 double mutation of the *E. coli rec*N promoter (*rec*N3-4) is cloned upstream of *lux*CDABE	This work

that are conserved in all LexA binding sites from *E. coli* and *S. typhimurium*, into GAA. The base pairs in italics at the 5′-end of primer 3 do not fit the wild-type DNA sequence and are chosen to introduce an *Eco*RI restriction site. This primer must be used with primer recN1 for PCR amplification.

To introduce a 'promoter up' mutation, primer recN4 was chosen from positions 294 to 331 with sequence AAAA*GAATTC*TAATTTTACGCCAGCCTCTT**G**ACTGTAT. This primer introduces a G (bold) at the consensus position of the promoter −35 region (underlined), and also introduces an *Eco*RI restriction site at the 5′-end of the promoter region. Primer recN4 must be used in combination with primer recN2 for PCR amplification. By using the combination of primers recN3 and recN4 for PCR amplification, a 'promoter up' mutation plus deletion of the LexA2 binding site from the *rec*N promoter can be obtained.

The wild-type promoter region of the *rec*N gene as well as its mutation derivatives were obtained using the sets of primers described above. This resulted in the following PCR fragments:

- recN1-2, wild type *rec*N promoter;
- recN1-3, *rec*N promoter lacking the LexA2 site;
- recN2-4, *rec*N promoter with promoter up mutation;
- recN3-4, *rec*N promoter with promoter up mutation lacking the LexA2 site.

These PCR fragments were all digested with *Eco*RI and cloned in *Eco*RI linearized pMOL877. This resulted in the plasmids pMOL1066 (recN1-2), pMOL1067 (recN1-3), pMOL1068 (recN2-4) and pMOL1069 (recN3-4), whose restriction maps are shown in Fig. 1. The plasmids were transformed to *E. coli* ED8739 (Met^-, $RecA^+$) [18] using electroporation. Transformants were selected for growth on tetracycline, toothpicked in duplo onto 869 medium

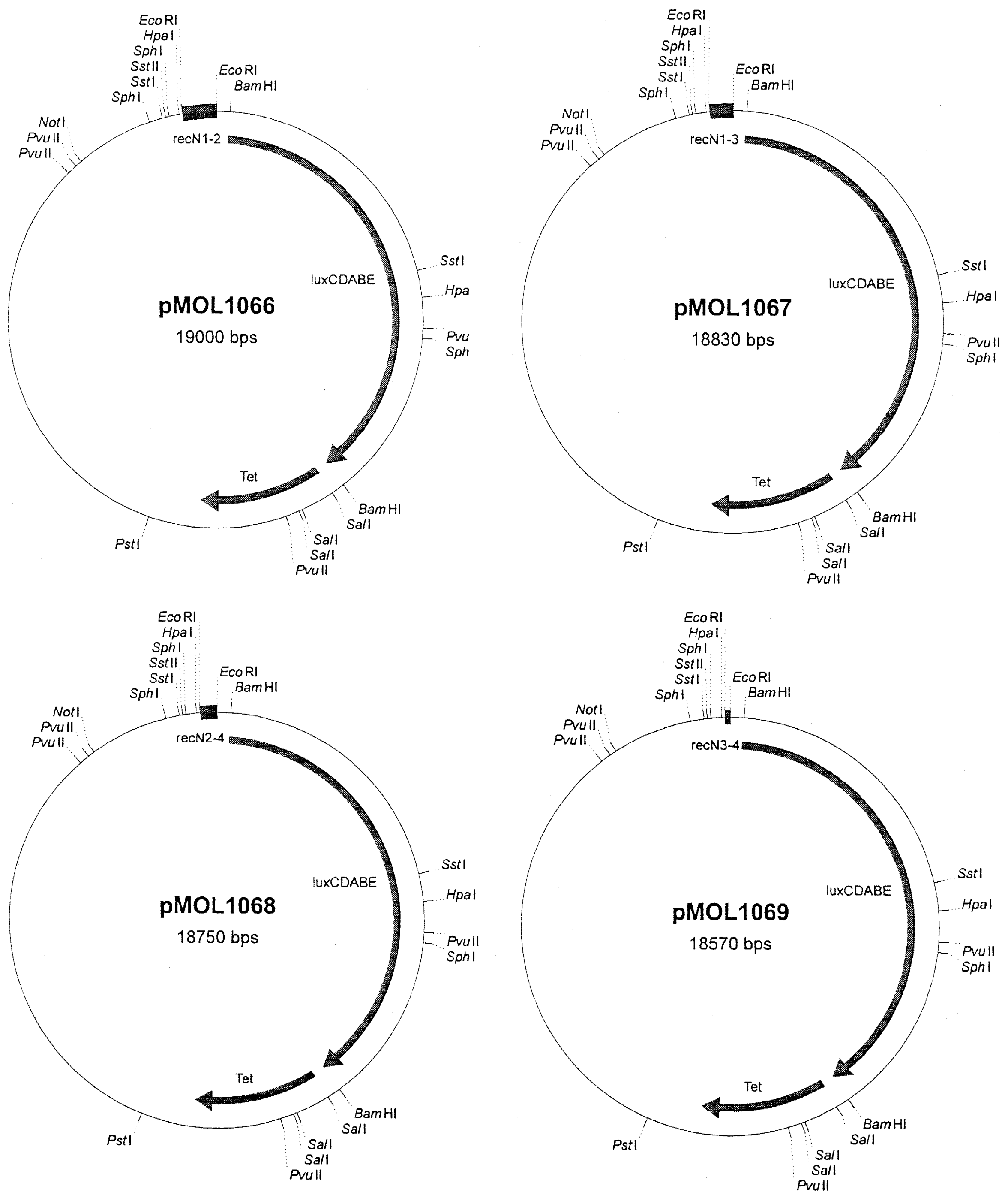

Fig. 1. Restriction maps of the promoter probe vector pMOL877 derivatives pMOL1066, pMOL1067, pMOL1068 and pMOL1069 in which the *rec*N promoter (pMOL1066, recN1-2), a *rec*N promoter with an inactivated LexA2 site (pMOL1067, recN1-3), a *rec*N promoter with a promoter up mutation (pMOL1068, recN2-4) and a *rec*N promoter with both the promoter up mutation and the inactivated LexA2 site (pMOL1069, recN3-4) are cloned. The positions of the promoterless *lux*CDABE operon of *V. fischeri*, the Tc^R marker (Tet), and the most important restriction sites are indicated.

containing tetracycline, and subsequently one plate was irradiated with UV. Positive clones that showed UV inducible light production were visualised using autoradiography. The plasmid content of the positive clones was examined using both PCR and restriction analysis. In order to construct the recombinant Ames test strains, the different plasmid constructs were introduced by electroporation in the *S. typhimurium* strains TA98, TA100 and TA104.

2.3. Luminometry for the testing of genotoxicity with the luminescent strains

To prepare the test cultures, 5 ml 869 medium was inoculated with the required strain and grown overnight (ON) at 37°C on a rotative shaker. Next morning, 20 μl of the ON culture was diluted in 2.5 ml 869 medium and incubated for 1 h at 37°C with shaking. Subsequently, the culture was diluted 10 times in 869 medium and 90 μl of the dilution was added per well of a 96-well microtiter plate that already contained 10 μl of the test product solution. If S9 mix was required for metabolic activation, the culture was diluted 10 times in 869 medium that contained 30% (v:v) S9 mix.

The 96-well plate was placed into the Microlumat LB96P luminometer (EG and G Berthold) and measuring was performed with the following parameters: 1 s/well; cycle time 5 min; 60 cycles; incubation temperature 30°C. After completing the measurements, the data were transferred into an Excel macro sheet and the signal-to-noise ratio, being the light production of induced cells divided by the light production of non-induced cells, was calculated for each measurement. A compound was considered genotoxic when the signal-to-noise ratio was equal or higher than 2 for at least two concentrations and when a clear dose–effect response was observed.

2.4. Genotoxicity and mutagenicity testing

Genotoxicity testing using the SOS chromotest was performed using the protocol of Quillardet and Hofnung [19]. For mutagenicity testing with the Ames test strains TA98, TA100 and TA104, the procedure described by Maron and Ames [7] was used.

2.5. Compounds used for genotoxicity testing

The following compounds were used for genotoxicity testing: 2-aminofluorene (Janssen Pharmaceutica, CAS No. 153-78-6); Benzo[*a*]pyrene (Sigma, CAS No. 50-32-8); chrysene (Sigma, CAS No. 218-01-9); H_2O_2 (J.T. Baker Chemical Co., Phillipsburg, NY, USA, CAS No. 7722-84-1); ICR191 Acridine (Sigma, CAS No. 17070-45-0); $K_2Cr_2O_7$ (UCB, CAS No. 7778-50-9); MMS (Merck, CAS No. 66-27-3); novobiocine (Sigma, CAS No. 1476-53-5); 4-NQO (Sigma, CAS No. 56-57-5); 2,4,5,7-tetranitro-9-fluorenone (Sigma, CAS No. 746-53-2). As negative controls, casaminoacids (Difco) and $CdCl_2 \cdot 2^{1/2}\ H_2O$ (J.T. Baker Chemical Co.) were used.

3. Results

3.1. Luminescence testing in E. coli

To examine the inducibility of the different promoter constructs in pMOL1066 to pMOL1069 by the bacterial SOS system of *E. coli*, induction experiments were performed using 64 ppm of MMS. As control, non-induced cells were taken. The results as signal-to-noise ratio are presented in Fig. 2. From these results it can be concluded that:

- pMOL1066 (recN1-2), containing the wild-type *rec*N promoter, is less well expressed after SOS induction than the derivatives containing a mutated *rec*N promoter (also total light production is lower, result not shown);

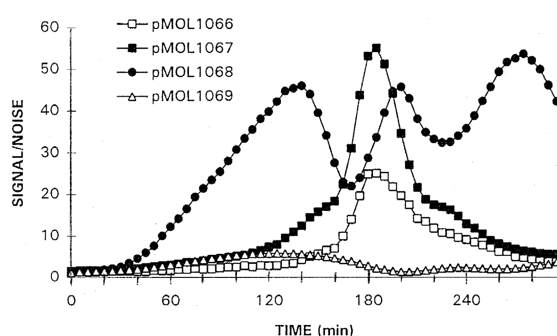

Fig. 2. Light induction kinetics presented as signal-to-noise ratio of *E. coli* 1106 containing pMOL1066, pMOL1067, pMOL1068 or pMOL1069 challenged with 64 ppm MMS. The light production was measured during 5 h.

- pMOL1067 (recN1-3), containing the *rec*N promoter lacking the LexA2 site, is very well induced and expressed after SOS induction. The induction profile is very similar to the one observed with pMOL1066, but the signal-to-noise values are much higher;
- pMOL1068 (recN2-4), containing the *rec*N promoter with promoter up mutation, is very well induced and expressed after SOS induction. The total light production with this construct as well as the initial induction kinetics are faster than those observed with pMOL1067. However, over a longer period the induction pattern as signal noise/ratio is more fluctuating than for pMOL1067;
- pMOL1069 (recN3-4), containing the *rec*N promoter with promoter up mutation lacking the LexA2 site, has a very strong light production but a poor signal-to-noise ratio. Therefore, this construct is not suitable as a genotoxicity biosensor.

In another series of experiments, the dose–effect curves for *E. coli* 1106 containing pMOL1066, pMOL1067, pMOL1068 or pMOL1069 were determined for MMS (concentrations from 0.5 to 128 ppm) and UV irradiation (irradiation time of 2–10 s). The results of these experiments are presented in the Fig. 3A, B.

In both experiments, the best results were obtained with pMOL1068 (recN2-4), while pMOL1069 (recN3-4) gave the lowest signal-to-noise ratios.

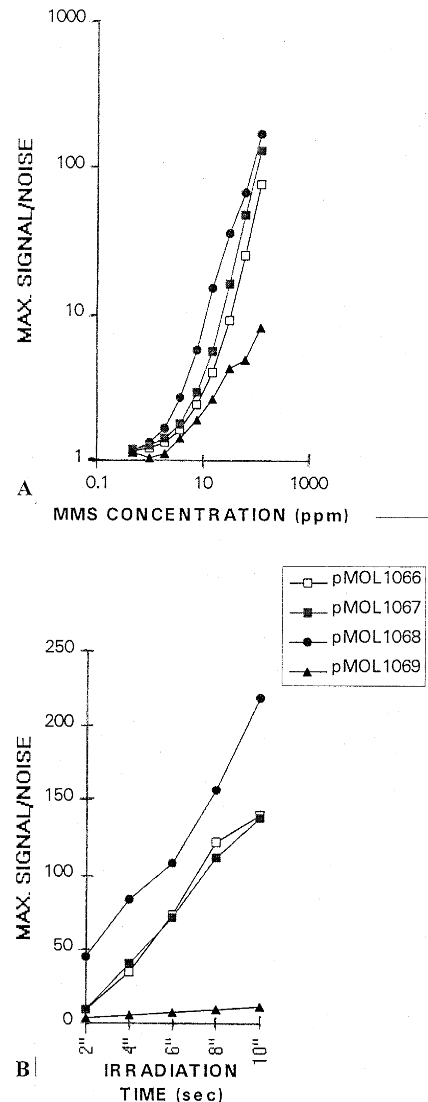

Fig. 3. Dose–effect curves for MMS (concentrations from 0.5 to 128 ppm) (A) and dose–effect curves for UV (irradiation time from 2 to 10 s) (B) presented as maximal signal-to-noise ratio against concentration or irradiation time for *E. coli* 1106 containing pMOL1066, pMOL1067, pMOL1068 or pMOL1069. The light production was measured during 5 h.

3.2. Mutagenicity and genotoxicity testing in *S. typhimurium*

Plasmids pMOL1066 to pMOL1069 were introduced in the Ames test *S. typhimurium* strains TA98, TA100 and TA104. In order to examine whether the introduction of pMOL1066, pMOL1067, pMOL1068 or pMOL1069 had any effect on the characteristics of the *S. typhimurium* strains for mutagenicity testing (determined as the reversion frequency from His^- to His^+ phenotype in the presence and absence of a mutagenic product), standard Ames tests were performed with both the original Ames test strains and their derivatives containing the above-mentioned plasmids. Testing was performed on MMS, 4-NQO, furazolidone, 2-AF and benzo[*a*]pyrene. Only the results for 4-NQO are presented (Table 2A, B), but similar results were obtained for the other tested compounds. By comparing the results from Table 2A and B it can be concluded that the introduction of the plasmids did not change the His-reversion characteristics of the recombinant strains.

To examine the inducibility of the different *E. coli rec*N promoter constructs by the bacterial SOS

Table 2
Ames test performed on 4-NQO with the *S. typhimurium* strains TA98, TA100 and TA104 (A) and MA985, MA988 and MA989 containing pMOL1068 (B)

Product and quantity	Number of His$^+$ revertants					
	TA98		TA100		TA104	
(A)						
4-NQO, 1.00 μl	310	348	2288	2160	1520	1760
4-NQO, 0.10 μl	69	60	400	440	544	520
4-NQO, 0.01 μl	47	50	182	174	272	448
Average blanks	38		131		359	
Average blanks with DMSO	33		125		374	
Conclusion	+		+		+	
	MA985		MA988		MA989	
(B)						
4-NQO, 1.00 μl	138	189	482	461	527	691
4-NQO, 0.10 μl	27	37	112	101	149	161
4-NQO, 0.01 μl	8	15	76	86	93	106
Average blanks	32		69		95	
Average blanks with DMSO	35		73		102	
Conclusion	+		+		+	

Strains MA985, MA988 and MA989 are based on the genetic background of TA98, TA100 or TA104, respectively.

system of *S. typhimurium*, induction experiments were performed using 64 ppm of MMS or 25.6 ppb 4-NQO. As control, non-induced cells were taken. The results as signal-to-noise ratio obtained for the *rec*N–*lux* fusions pMOL1067 and pMOL1068 are presented in Fig. 4A, B. From these figures it can be concluded that the induction kinetics with pMOL1068 are slightly faster than those observed for pMOL1067, but that the maximum signal-to-noise ratios are higher with pMOL1067 than with pMOL1068. Therefore, both constructs are of interest and complementary for genotoxicity testing.

The minimal detectable concentrations (MDC, in nmol/assay) were determined for 13 compounds with *S. typhimurium* Ames test strains containing pMOL1066, pMOL1067, pMOL1068 or pMOL1069. The results with *S. typhimurium* TA104 containing pMOL1066, pMOL1067, pMOL1068 or pMOL1069, and *S. typhimurium* TA98 pMOL890, pMOL1067 or pMOL1068 are presented in Table 3. In addition, MDC values found with the SOS chromotest and the Ames test are also presented. For certain compounds, literature MDC values were used. Here, it was sometimes impossible to calculate the Ames test MDC values. For these products a positive (+) or negative (−) response is indicated. Two products, glucose and $CdCl_2$, were chosen as negative controls.

From Table 3, it can be concluded that the greatest sensitivity was obtained with the strains containing pMOL1067 or pMOL1068 (see also Table 1). The MDC values obtained with all constructs were always lower than those found with the SOS chromotest, except for the results with ICR191, which

Table 3
Minimal detectable concentrations (MDC, in nmol/assay) determined for 13 products, with *S. typhimurium* strains MA999, MA984, MA989, MA1001 (TA104 background) and MA980 and MA985 (TA98 background)

Product	MDC (nmol/assay)							
	MA999	MA984	MA989	MA1001	MA980	MA985	SOS chromotest	Ames test
2-Aminofluorene	0.44	0.22	0.22	0.22	0.44	0.22	3.56	0.67
2,4,5,7-Tetranitro-9-fluorenone	0.022	0.011	0.022	0.011	0.022	0.011	0.24	0.001
$K_2Cr_2O_7$	21.8	5.4	1.4	10.9	5.4	1.4	136	91
H_2O_2	11.8	11.8	11.8	5.9	1.5	1.5	23	−
Novobiocine	1.26	0.32	1.26	0.32	0.079	0.63	−	+
ICR191	1.1	0.55	0.55	1.1	0.28	0.14	0.13	0.7
Chrysene	1.095	0.55	1.095	0.55	5.5	5.5	11	4.4
Glucose	−	−	−	−	−	−	−	−
Furazolidone	0.00044	0.00022	0.00022	0.00044	0.00176	0.00176	0.01	0.1
Benzo[*a*]pyrene	0.16	0.079	0.16	0.16	0.16	0.16	0.7	4
4-Nitroquinoline-*N*-oxide	0.0034	0.0017	0.0008	0.0017	0.0017	0.0008	0.006	0.003
Methylmethane sulfonate	7.3	7.3	7.3	7.3	7.3	7.3	20	60
$CdCl_2$	−	−	−	−	−	−	−	−

MDC values are also presented for the SOS chromotest and the Ames test. −, negative.

Table 4
Comparison between the VITO-TOX test, the Ames test and the SOS chromotest

Agent	VITO-TOX	Ames	SOS	Group	Remarks
Drugs					
ICR191	+	+	+	acridine	
Cyclophosphamide	−	+	−	antineoplastic drug	
Mitomycin C	+	−	+	antineoplastic drug	
Methotrexate	+	+	+	antineoplastic drug	
Antimicrobials					
Furazolidone	+	+	+	antimicrobial	
Novobiocine	+	+	−	antimicrobial	
Carbadox	+	+	+	antimicrobial	
Nifuroxazide	+	+	+	antimicrobial	
Nalidixic acid	+	−	+	antimicrobial	
Pesticides, herbicides					
Ethylenethiourea	−	+	−	pesticide	
Carbaryl	+	+	+	pesticide	
Lindane	−	−	−	pesticide	
Pentachlorophenol	+	+	+	herbicide	
Inorganic agents					
SeO_2	+	+	nd	metal	oxidizing agent
$ZnCl_2$	−	−	−	metal	industrial
$CdCl_2$	−	−	−	metal	industrial
$K_2Cr_2O_7$	+	+	+	metal	oxidizing agent
H_2O_2	+	−	+	peroxide	oxidizing agent
Sodium azide	+	+	−	azide	industrial
PAHs					
Benzo[*a*]pyrene	+	+	+	PAH (pentacyclic)	env. pollutant
Chrysene	+	+	+	PAH (tetracyclic)	env. pollutant
Naphthalene	−	−	−	PAH (tetracyclic)	env. pollutant
Pyrene	+	+	−	PAH (tetracyclic)	env. pollutant
2,4,5,7-Tetranitro-9- fluorenone	+	+	+	PAH (tricyclic)	env. pollutant
4-Nitroquinoline-*N*-oxide	+	+	+	PAH (tricyclic)	env. pollutant
Fluoranthene	+	+	+	PAH (tricyclic)	env. pollutant
Anthracene	−	−	−	PAH (tricyclic)	env. pollutant
Phenanthrene	+	−	+	PAH (tricyclic)	env. pollutant
Laboratory chemicals					
N-Nitrosodiethylamine	+	+	+	nitrosamine	
2-Aminofluorene	+	+	+	aromatic amine	
Glucose	−	−	−	sugar	
Sodium dodecyl sulfate	−	−	−	anionic detergent	
Glucose	−	−	−	sugar	
Methylmethanesulfonate	+	+	+	sulfonate	
Ethylmethanesulfonate	+	+	+	sulfonate	
Solvents, fuel, etc.					
Hydrazine	+	+	+	hydrazine	rocket fuel
Epichlorohydrine	+	+	+	epoxypropane	solvent
Electromagnetic waves					
UV-rays	+	+	nd	electromagnetic	therapeutic, sun, etc.
X-rays	+	+	+	electromagnetic	therapeutic

+, genotoxic; −, not genotoxic; nd, not determined; env., environmental.

were similar. The results were also more sensitive than those found with the Ames test, except for 2,4,5,7-tetranitro-9-fluorenone. Therefore, it can be concluded that the use of the *rec*N-lux gene fusions (pMOL1066, pMOL1067, pMOL1068 or pMOL1069) in the *S. typhimurium* Ames test strains

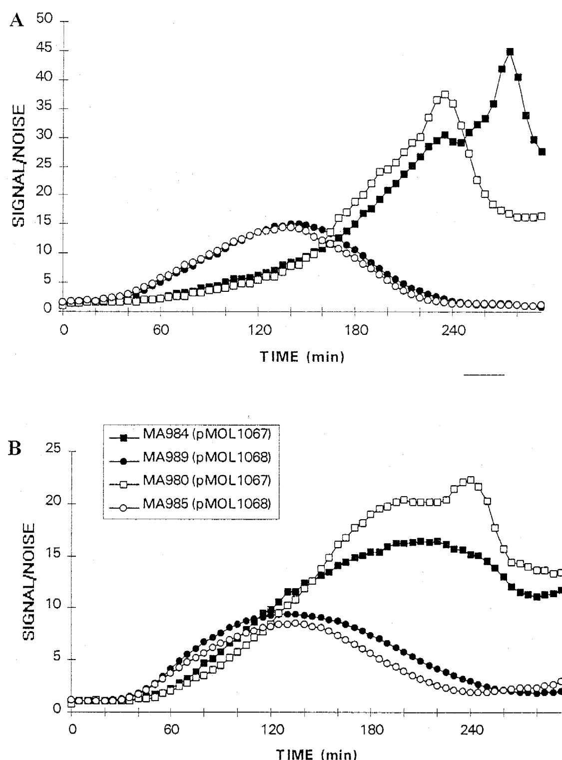

Fig. 4. Light induction kinetics presented as signal-to-noise ratio of *S. typhimurium* MA980, MA984, MA985 and MA989 challenged with 64 ppm MMS (A) or 25.6 ppb 4-NQO (B). The light production was measured during 5 h.

provides a more sensitive way for detecting genotoxicity then the SOS chromotest and the Ames test.

Table 4 presents the results of genotoxicity tests performed on different classes of agents with the TA98 and TA104 strains containing pMOL1067 or pMOL1068. Since the results found with these strains were similar, only the final outcome of the tests is presented (positive or negative). Similar results are given for the SOS chromotest (SOS) and the Ames test. It can be concluded from Table 4 that the hybrid TA Ames test strains could detect genotoxicity for all categories of products tested, and that only a few differences are observed when the results of the SOS chromotests and the Ames tests are compared.

When a product is negative in the tests with strains TA98 and TA104 containing pMOL1067 or pMOL1068, it is also negative with the SOS chromotest. However, novobiocine, sodium azide and pyrene, which are potentially genotoxic, were negative in the SOS chromotest, but positive in the test with the TA98 and TA104 strains containing pMOL1067 or pMOL1068. These results suggest that the tests with strains TA98 and TA104 containing pMOL1067 or pMOL1068 are more sensitive and accurate for genotoxicity testing than the SOS chromotest.

When a product is negative in the tests with strains TA98 and TA104 containing pMOL1067 or pMOL1068, it is also negative with the Ames test, this with the exception of cyclophosphamide and ethylene thiourea. This is not surprising, since these potentially mutagenic products are known not to induce the bacterial SOS response.

False-negative genotoxic products in the Ames test, such as mitomycin C, naladixic acid, hydrogen peroxide, and phenanthrene, were found to be positive in the test with the TA98 and TA104 strains containing pMOL1067 or pMOL1068. Therefore one can conclude that the test with the TA98 and TA104 strains containing pMOL1067 or pMOL1068 is at least as accurate as the Ames test.

3.3. Genotoxicity testing on combinations of products

It was examined how the different constructs would react when more than one genotoxic product

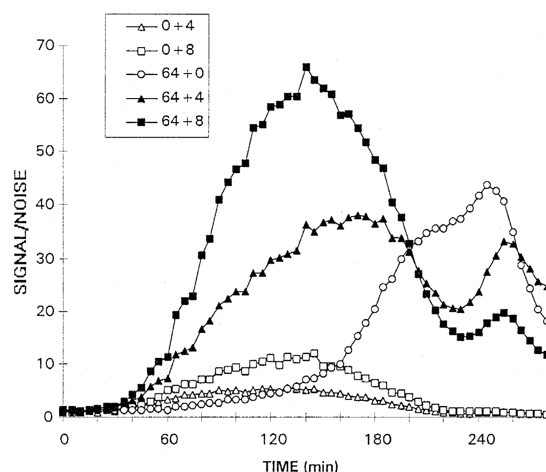

Fig. 5. Light induction kinetics presented as signal-to-noise ratio of *S. typhimurium* MA980 challenged with combinations of different concentrations (in ppm) of MMS and novobiocine. In the key, the concentration of MMS is first indicated, followed by the concentration of novobiocine.

is present in the test sample. This is important, since environmental samples often contain more than one genotoxic pollutant. Also pharmaceutical products might be combinations of different active genotoxic substances. The experiments were performed with MA980 and MA984 (TA98 and TA104 containing pMOL1067, respectively). These strains were chosen since they show similar induction kinetics and the best signal-to-noise ratios. (see Fig. 4A, B). As test products, several combinations of MMS and novobiocine were taken. These compounds were used since they were found before to have different induction kinetics (e.g., MMS gives a maximum after 2 h, while the maximal signal-to-noise ratio with novobiocine is observed after 4 h). The results obtained with strain MA980 are presented in Fig. 5. Similar results were obtained with strain MA984.

From these results it can be concluded that with strains containing pMOL1067 (*rec*N1-3::*lux*) the presence of at least two individual genotoxic compounds can be determined in a mixture of genotoxic compounds. This is not obvious, because one would expect an increase of the maximum signal instead of two individual maxima.

3.4. Toxicity testing

The different constructs produce, in the absence of a genotoxic product, a background light signal which is called the noise. A decrease in light production as compared to the noise signal can be used to determine toxicity instead of genotoxicity. Strains containing pMOL1068, which show a relatively high noise signal, could be very suitable for toxicity testing (their noise increases in the course of a 4-h experiment from 100 to 13 000 relative light units), while strains containing pMOL1066 or pMOL1067 that show a low noise signal are of less use (their noise increases in the course of a 4-h experiment from 20 to 750 relative light units). Toxicity testing is illustrated in Fig. 6, where a concentration-dependent decrease in light production was observed for strain MA985 (pMOL1068) when challenged with different concentrations of $CdCl_2$.

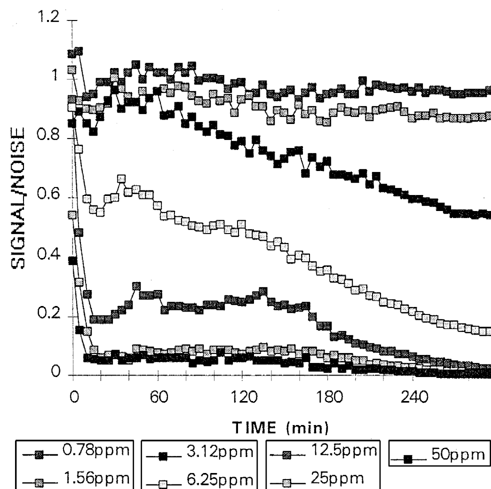

Fig. 6. Decrease in time of the signal-to-noise ratio found for MA985 when the strain is incubated with different concentrations (in ppm) of $CdCl_2$.

4. Discussion

Four SOS-responsive gene fusions were constructed that are based on the *E. coli rec*N promoter. The *rec*N promoter region, which resembles a σ70-like promoter, contains two LexA binding sites [17], the first site (*lex*A1) overlapping with the −35 region of the promoter and the second site (*lex*A2) overlapping with the −10 region of the promoter. The construct based on the wild-type *rec*N promoter, pMOL1066, and pMOL1067 that is based on the *rec*N promoter with a deleted *lex*A2 site, showed similar induction kinetics. For both constructs, a maximum in bioluminescence (as S/N ratio) was observed with MMS after 3 h. Finch et al. [15] showed that the maximum synthesis of the RecN protein occurred within 30 min of DNA damage after treatment with MMC. In our system, maximum bioluminescense was observed after 2–4 h, depending on the compound tested. This difference is most likely due to the nature of the *lux* expression system.

The maximum S/N value for pMOL1067 was considerably higher than the value found for pMOL1066 (56 as compared to 25). This indicates that despite the deletion of the *lex*A2 site the *rec*N1-3 promoter mutant remains tightly regulated by LexA.

A much faster induction was observed with pMOL1068, that is based on the *rec*N2-4 promoter with the promoter-up mutation. Probably due to a better binding of the RNA-polymerase to the mutated σ70-like promoter sequence, both faster and

also higher induction levels are observed. However, a construct containing a mutated *rec*N promoter with both the promoter-up mutation and deletion of the *lex*A2 site is only weakly regulated by the bacterial SOS response: this construct resulted in a high and nearly constitutive expression of the *lux* operon.

The *S. typhimurium* strains containing pMOL1067 or pMOL1068 were the most sensitive and fastest strains for the detection of genotoxic compounds. These strains were used to develop a genotoxicity test that we refer to as the VITOTOX test. The VITOTOX test strains were validated for several classes of genotoxic agents, such as drugs, antimicrobials, pesticides, herbicides, inorganics, polycyclic aromatic hydrocarbons (PAHs), laboratory chemicals, solvents, fuel and electromagnetic waves. The hybrid strains were able to detect genotoxicity for all categories of products tested. Only a few differences were observed with the results found for the SOS chromotest and the Ames test, but a better correlation was observed with the results from the Ames tests than with the results from the SOS chromotest.

The VITOTOX test has the following features and advantages over the Ames test and the SOS chromotest: the VITOTOX test allows mutagenicity to be detected by the same procedure as for the Ames test, or genotoxicity (as for the SOS chromotest) and toxicity using luminometry. Toxicity can be determined in 20–30 min. Genotoxicity can be detected within 2–4 h, which is as fast as the SOS chromotest. Since neither plating nor disruption of the cells is required to measure genotoxicity, as is the case for the SOS chromotest, the kinetics of genotoxicity can be determined, as well as the presence of multiple genotoxic compounds within the same sample. In addition, the VITOTOX test strains provide a more sensitive way of detecting genotoxicity than the SOS chromotest and the Ames test, as for most products tested, lower or similar MDC values were found.

Interestingly, the results found with the VITOTOX test for environmental pollutants like PAHs corresponded more closely with the results reported for the Ames test than with the results reported for the SOS chromotest [20]. This was unexpected, because both the VITOTOX test and the SOS chromotest test strains contain promoter– reporter gene fusions that are regulated by the bacterial SOS response. Therefore, the VITOTOX test can be very useful for the evaluation of the bioremediation of PAH-contaminated soils, as well as for the assessment and evaluation of sites and waters polluted with other chemicals. At present, such studies are being carried out in collaboration with other institutes.

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